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Mouse models of rhinovirus-induced disease and exacerbation of allergic airway inflammation

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Abstract

Rhinoviruses cause serious morbidity and mortality as the major etiological agents of asthma exacerbations and the common cold. A major obstacle to understanding disease pathogenesis and to the development of effective therapies has been the lack of a small-animal model for rhinovirus infection. Of the 100 known rhinovirus serotypes, 90% (the major group) use human intercellular adhesion molecule-1 (ICAM-1) as their cellular receptor and do not bind mouse ICAM-1; the remaining 10% (the minor group) use a member of the low-density lipoprotein receptor family and can bind the mouse counterpart. Here we describe three novel mouse models of rhinovirus

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infection: minor-group rhinovirus infection of BALB/c mice, major-group rhinovirus infection of transgenic BALB/c mice expressing a mouse-human ICAM-1 chimera and rhinovirus-induced exacerbation of allergic airway inflammation. These models have features similar to those observed in rhinovirus infection in humans, including augmentation of allergic airway inflammation, and will be useful in the development of future therapies for colds and asthma exacerbations.

Rhinoviruses cause the common cold, hospitalization of infants¹, pneumonia in the immunosuppressed² and the majority of acute exacerbations of asthma^{3–6} and chronic obstructive pulmonary disease (COPD)⁷. These diseases generate hundreds of billions of dollars in healthcare costs⁸, and the morbidity and mortality attributable to rhinovirus infections is enormous. No effective treatment is available.

Attempts to develop small-animal models of rhinovirus infection in many species, from mice to monkeys, have failed, and an intracellular block to rhinovirus replication in mouse cells was proposed to be the cause⁹. The lack of small-animal models of rhinovirus infection has thus severely hampered mechanistic insight into and therapeutic development for rhinovirus infections over the past 50 years.

Ten percent of the ~100 rhinovirus serotypes make up the minor receptor group of viruses, which can use both the human and mouse forms of the receptor to enter cells of either species¹⁰. The remaining 90% comprise the major receptor group and use human ICAM-1 for cell attachment and entry¹¹, but these viruses do not bind mouse ICAM-1 (refs. 12,13). Here we report the development of *in vivo* mouse models of major- and minor-group rhinovirus infection and of rhinovirus-induced asthma exacerbation.

RESULTS

A mouse model of minor-group rhinovirus infection

We inoculated BALB/c mice with 5×10^6 50% tissue-culture infective dose (TCID₅₀) of minor-group rhinovirus-1B, UV-inactivated rhinovirus-1B or major-group rhinovirus-16 (Fig. 1). Only rhinovirus-1B induced rapid neutrophilic inflammation, detectable at 8 h, peaking 1–2 d after infection (with neutrophils representing 60–70% of total bronchoalveolar lavage (BAL) cells) and returning to baseline levels by day 4 (Fig. 1a). A prolonged increase in lymphocytes detectable at day 2 and peaking 4–7 d after infection (~20% of BAL cells) was also observed only in rhinovirus-1B-infected mice (Fig. 1a). Macrophage numbers remained unchanged in all groups (Supplementary Fig. 1a online). Examination of stained lung sections revealed areas of extensive peribronchial and perivascular cellular infiltration (Fig. 1b) that were not detected in the lungs of control mice. Induction of mucin-5, subtypes A and C (Muc5AC), and Muc5B mRNA in lung (Fig. 1c,e), Muc5AC and Muc5B protein in BAL (Fig. 1d,f) and staining for Muc5AC and Muc5B in bronchial epithelium (Supplementary Fig. 1b) were observed only in rhinovirus-1B-infected mice, with Muc5AC protein abundance peaking at day 1 and remaining elevated at day 7 and Muc5B elevated throughout days 1–14 after infection. Rhinovirus-1B induced the early production of the neutrophil chemokines macrophage inflammatory protein-2 (MIP-2) and KC (Fig. 2a,b) concurrently with the onset of neutrophilia (Fig. 1a) and also induced the dendritic and T cell chemokine MIP-3α (CCL20, refs. 14,15); Fig. 2c), whereas the T cell chemokines IP-10 (CXCL10), RANTES (CCL5) and I-TAC (CXCL11) were induced later, peaking at 1–2 d (Fig. 2d–f) and preceding the lymphocytosis on days 2–7 (Fig. 1a). Rhinovirus-1B induced the proinflammatory cytokines tumor necrosis factor-α (Supplementary Fig. 1c), interleukin-6 (IL-6) and IL-1β between 8 and 48 h after infection

(Fig. 2g,h). Thymic stromal lymphopoietin, IL-4, IL-13 and IL-17 were all undetectable (data not shown).

The levels of rhinovirus-1B genomic RNA in lung and of infectious virus particles in BAL from 8 to 24 h after infection were significantly increased compared to those in mice treated with UV-inactivated virus (Fig. 3a,b). Viral replication was further shown by *in situ* hybridization using an antisense RNA probe to detect genomic viral RNA and a sense RNA probe to detect replicative viral RNA (Fig. 3c). Strong staining for replicative RNA was detectable in the airway epithelium 8 h after infection (data not shown), peaking at 12 h (Fig. 3c) and becoming undetectable by 48 h (data not shown), whereas genomic RNA peaked at 24 h (Fig. 3c) and was observable until 72 h after infection (data not shown). Rhinovirus-1B induced interferon- α (IFN- α), IFN- β and IFN- λ proteins in BAL fluid (Fig. 3d); IFN- β was produced earliest at 8 h. All IFNs peaked at 24 h, and IFN- α and IFN- β had returned to baseline by 48 h (Fig. 3d), whereas IFN- λ remained significantly elevated at this time (Fig. 3d).

IFN- γ production was increased in lung leukocytes from rhinovirus-1B-infected mice compared to those from rhinovirus-16-infected mice (Fig. 3e). IFN- γ production at day 4 was due to nonspecific activation of leukocytes, as only leukocytes obtained at day 7 produced significantly more IFN- γ when re-stimulated *ex vivo* with rhinovirus-1B (Fig. 3e), which is consistent with induction of a virus-specific acquired immune response. Rhinovirus-1B-specific IgG titers were significantly higher at day 7 than at day 0 and were further increased at day 10 in rhinovirus-1B-infected mice compared to those in mice infected with UV-inactivated virus (Fig. 3f).

A transgenic mouse model of major-group rhinovirus infection

We generated a BALB/c mouse strain transgenic for the rhinovirus-binding extracellular domains 1 and 2 of human ICAM-1 (Supplementary Methods and Supplementary Fig. 2a online). Rhinovirus-16 infection of transgenic mice, but not rhinovirus-16 infection of nontransgenic BALB/c controls or UV-inactivated rhinovirus-16 infection of transgenic mice, resulted in outcomes similar to those observed with the rhinovirus-1B infection model, including BAL neutrophilia and lymphocytosis (Fig. 4a); increased Muc5B protein in BAL (Fig. 4b); increased viral RNA levels (Fig. 4c); and induction of IFNs (Fig. 4d), chemokines, IL-1 β (Fig. 4e–g) and virus-specific antibodies (Supplementary Fig. 2b).

Rhinovirus exacerbation of allergic airway inflammation

Finally, to develop a mouse model of rhinovirus-induced asthma exacerbation, we challenged BALB/c mice sensitized to ovalbumin (OVA) with OVA or PBS control. During the challenge, mice were also inoculated with either rhinovirus-1B or UV-inactivated rhinovirus-1B. The combination of OVA challenge and rhinovirus-1B infection (RV-OVA) resulted in a significant increase in the BAL of neutrophils on day 1 (Fig. 5a) and eosinophils on day 7 (Supplementary Fig. 3a online), and a prolonged increase in lymphocytes in BAL from days 3 to 14 (Fig. 5a), compared with mice infected with virus but not challenged (RV-PBS) and allergen-challenged mice dosed with UV-inactivated virus (UV-OVA). In addition to increased airway inflammation, RV-OVA mice also showed significantly enhanced airway hyper-responsiveness on day 1 compared with that observed in mice infected with virus alone or mice challenged with allergen alone, whether the hyper-responsiveness was measured non-invasively (Fig. 5b, left) or invasively (Fig. 5b, right). RV-OVA mice showed significantly increased Muc5AC protein abundance in BAL at day 7 and Muc5B expression at day 14 compared with UV-OVA-treated and RV-PBS-treated mice (Fig. 5c), as well as significant induction of IL-4 (day 1) and IL-13 (days 1 and 2) expression compared with UV-OVA controls (Fig. 5d,e), whereas IL-5 abundance

(Supplementary Fig. 3b) was not significantly increased. Significant induction of virus-specific IFN- γ was also observed in RV-OVA mice compared to those receiving either viral or allergen challenge alone (Fig. 5f).

DISCUSSION

Rhinoviruses, the cause of common colds, are the most frequent precipitants of acute exacerbations of asthma and COPD^{3–7}, as well as causes of other serious respiratory diseases^{1,2}. The mouse models of minor- and major-group rhinovirus infection reported herein are characterized by several relevant disease-related outcomes, including airway neutrophilic and lymphocytic inflammation^{16,17}, mucin secretion (a cardinal symptom of common colds that is also induced by rhinovirus infection of human airway epithelial cells *in vitro*¹⁸) and the induction of various chemokines and proinflammatory cytokines induced in human rhinovirus infections.

Our recent work has highlighted deficient induction of IFN- β and IFN- λ in lung cells from asthmatic donors in response to rhinovirus infection *ex vivo*^{19,20}. However, the relative importance of the IFN subtypes in rhinovirus infections is unknown, and although IFN- λ 1 has been shown to have antiviral effects^{21,22}, it has not been known whether IFN- λ s are produced by rhinovirus infections *in vivo*. Our data suggest that IFN- λ is the most sustained and abundant IFN subtype induced in the lung by rhinovirus infections.

The absence of pathologic, physiologic or antiviral responses in mice inoculated with UV-inactivated virus and in nontransgenic mice inoculated with rhinovirus-16 indicates that these responses were replication dependent. Replication was studied further with *in situ* hybridization, which showed early production of negative-sense replicative RNA and subsequent synthesis of viral genomic RNA in epithelial cells, which is consistent with the rhinovirus replication cycle. We observed further evidence of viral replication in the increased levels of rhinovirus-16 RNA in the lungs of transgenic mice compared to those in control mice and in the induction of type 1 IFN- α and IFN- β and type 3 IFN- λ s. The use of viruses better adapted for replication in mice^{23,24} or different mouse strains, including those with deficient IFN responses^{19,20}, may allow for better virus replication in mice.

Because rhinoviruses are the major precipitants of acute exacerbations of asthma^{3–6}, we studied the interactions between rhinovirus infection and allergic airway inflammation. In allergen-sensitized and allergen-challenged mice, rhinovirus infection exacerbated neutrophilic, eosinophilic and lymphocytic airway inflammation, airway hyper-responsiveness, mucus secretion and production of both T helper type 1 (T_H1) and T_H2 cytokines. These responses have been associated with rhinovirus-induced exacerbations of asthma²⁵, including fatal asthma²⁶, and are consistent with either rhinovirus infection substantially exacerbating allergic airway inflammation or, conversely, allergic airway inflammation substantially exacerbating rhinovirus-induced inflammation.

The mechanisms involved in the synergistic interaction between virus infection and allergen exposure to increase the risk of asthma exacerbations are unknown^{27,28}. T_H2-mediated inflammation is clearly implicated in allergic airway inflammation; however, it is not known whether rhinovirus infection can augment T_H2 responses to allergens. Conversely, virus infection is strongly associated with T_H1 responses, but it is not known whether allergic airway inflammation can augment rhinovirus-induced T_H1 responses. We show here that rhinovirus infection increases production of both IL-4 and IL-13 in response to allergen challenge, indicating that rhinovirus infection can exacerbate T_H2 responses to allergens. Similarly, allergen challenge increased rhinovirus induction of IFN- γ , confirming that allergen exposure can enhance T_H1 responses to rhinoviruses. Further studies will be

required to increase our understanding of these possible mechanisms of disease to aid the development of new therapeutic approaches.

In conclusion, these models of rhinovirus infection should be useful in investigating the pathogenesis and treatment of the common cold and acute exacerbations of asthma. With further development, they will probably provide a similar boost to research into exacerbations of COPD⁷ and other diseases in which rhinovirus infection is implicated².

METHODS

Mice and viruses

We purchased 6-week-old female BALB/c mice from Harlan. We generated human-mouse ICAM transgenic mice in house (Supplementary Fig. 2 and Supplementary Methods online). We grew major-group rhinovirus serotype 16 and minor-group rhinovirus serotype 1B in Ohio HeLa cells (European Collection of Cell Cultures). We obtained viruses from the American Type Culture Collection and passaged them five times in HeLa cells before purification. We titrated the viruses on HeLa cells by standard methods and inactivated them by exposing them to UV light at 1,200 mJ/cm² for 30 min. We lightly anesthetized the mice with isoflurane and infected them intranasally with 50 µl of rhinovirus.

TaqMan quantitative real-time PCR

We excised the left upper lobe (~15% of total lung) from each mouse, purified the RNA (RNeasy miniprep kit, Qiagen) and reverse-transcribed 5 µg using random hexamers (Omniscript RT kit, Qiagen) as primers. We normalized real-time PCR assays to 18S rRNA with Quantitect Probe PCR master mix (Qiagen) and primers and probe to human 18S rRNA²⁹. The TaqMan assays for measuring rhinovirus RNA have been described previously²⁰.

Asthma exacerbation model

We sensitized the mice with 50 µg OVA in 2 mg aluminum hydroxide intraperitoneally in a volume of 200 µl on day 1. We challenged lightly anesthetized (isoflurane) mice with 50 µg OVA (Calbiochem) in 30 µl of PBS (controls received PBS alone) on three consecutive days (days 10–12) to induce allergic airway inflammation. While they were under anesthesia during the third OVA challenge, we infected the mice intranasally with 50 µl rhinovirus-1B (2.5×10^6 TCID₅₀) or UV-inactivated rhinovirus-1B. We assessed lung function at 24 h after infection and killed the mice on the indicated days (Fig. 5). for end-point analyses.

Bronchoalveolar lavage

We cannulated mouse lungs through the trachea and then lavaged the lungs. We processed cellular fractions for differential staining by cytopinning. We analyzed BAL fluid for interferons, cytokines, chemokines and mucins by ELISA (R&D Systems) according to manufacturers' specifications.

Enzyme-linked immunosorbent spot

We carried out ELISpot analysis of IFN-γ production by purified lung leukocytes (harvested on day 4 or day 7 after infection). We cultured the cells for 3 d with or without *ex vivo* rhinovirus-1B stimulation. We read the plates with Leica Q Windows image software, quantifying the spot number per well.

Rhinovirus-specific antibody enzyme-linked immunosorbent assay

We coated plates (Maxisorp, Nunc) overnight with purified rhinovirus stock (2×10^8 TCID₅₀/ml). We incubated diluted serum samples for 1 h at 37 °C before detection with peroxidase-conjugated antibody to mouse IgG (Sigma).

Statistical analysis

All data were normally distributed and are expressed as means \pm s.e.m. We analyzed data by ANOVA and pinpointed differences between groups using Bonferroni's post-test, with 95% confidence levels (Prism 4, GraphPad) to determine significant differences. The numbers of mice used are indicated in the figure legends, and we repeated all experiments at least three times.

Further details on these and other methods are available in the Supplementary Methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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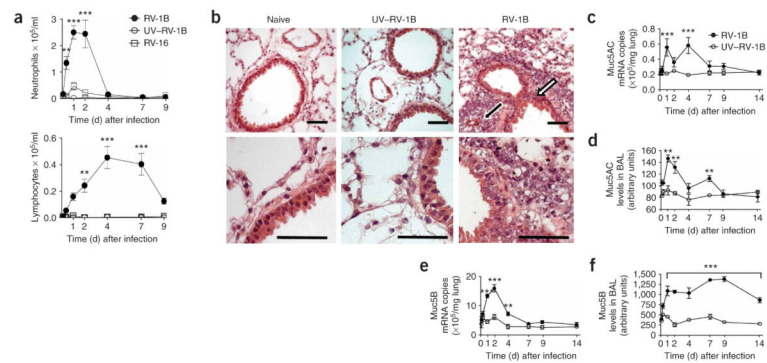


Figure 1.

Live minor-group rhinovirus-1B, but not UV-inactivated rhinovirus-1B or major-group rhinovirus-16, induces airway and lung inflammation and mucin production in BALB/c mice. Mice were infected with 5×10^6 TCID₅₀ of rhinovirus-1B (RV-1B), UV-inactivated rhinovirus-1B (UV-RV-1B) or rhinovirus-16 (RV-16). **(a)** Kinetic analysis of BAL cells showing neutrophils and lymphocytes at the indicated time points after infection, as assessed by differential cell counts. Representative of five experiments. Data are means for groups of four mice \pm s.e.m., ** $P < 0.01$ and *** $P < 0.001$ compared to both control groups. **(b)** Representative H&E-stained lung sections in naive mice (left column) or lungs taken at day 2 after infection with UV-RV-1B (center column) or RV-1B (right column). Perivascular (black arrow) and peribronchial (white arrow) inflammation are indicated. Scale bars, 50 μm . Images shown are typical of three mice. **(c)** Quantification of Muc5AC mRNA levels in lung tissue by quantitative RT-PCR. **(d)** Quantification of Muc5AC protein secretion in BAL by ELISA. The same analyses were carried out for Muc5B in **e** and **f**. Data are means \pm s.e.m., ** $P < 0.01$ and *** $P < 0.001$ compared with UV-RV-1B.

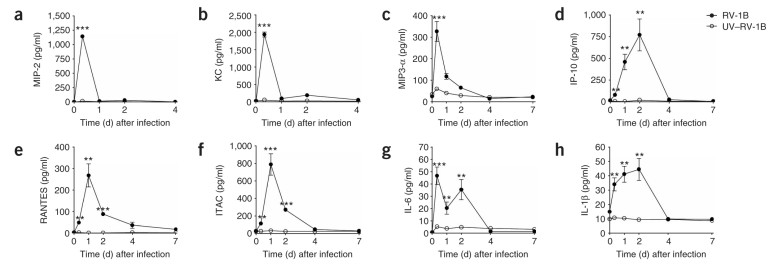


Figure 2.

Rhinovirus-1B induces neutrophil, dendritic cell and lymphocyte chemoattractant chemokine production and proinflammatory cytokine production. (a–h) Groups of four BALB/c mice were infected with 5×10^6 TCID₅₀ of RV-1B or UV-RV-1B. BAL was collected at 8 h and 1, 2, 4 and 7 days after infection, and cell-free BAL fluid was analyzed by quantitative ELISA for neutrophil chemoattractant chemokines MIP-2 (a) and KC (b); dendritic cell chemokine MIP-3 α (c); lymphocyte chemokines IP-10 (d), RANTES (e) and I-TAC (f); and the proinflammatory cytokines IL-6 (g) and IL-1 β (h). ** $P < 0.01$ and *** $P < 0.001$ for RV-1B compared with UV-inactivated control. Results are expressed as means \pm s.e.m.

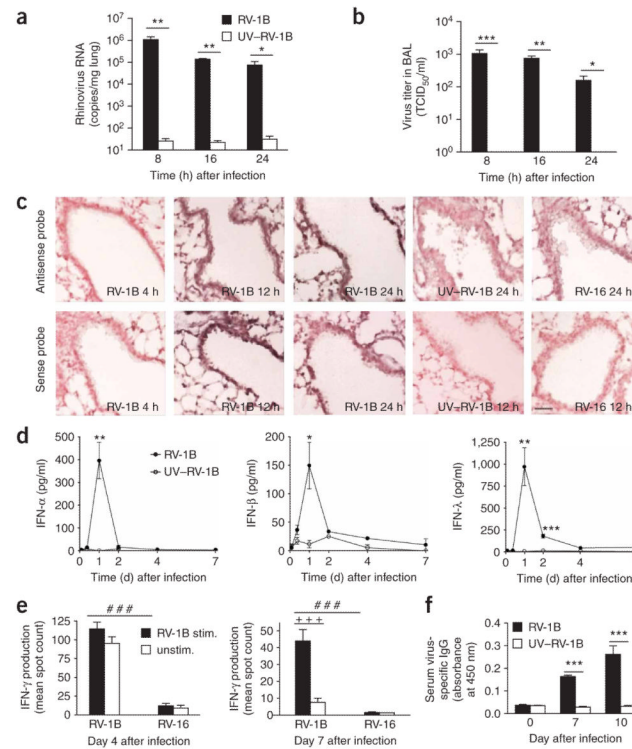


Figure 3.

RV-1B replication and induction of innate (antiviral IFN) and acquired virus-specific cellular and humoral immune responses in BALB/c mice. Groups of four mice were inoculated with 5×10^6 TCID₅₀ of RV-1B or UV-RV-1B. (a) Quantification of rhinovirus RNA in lung homogenates by quantitative RT-PCR. (b) Quantification of rhinovirus viral load in total BAL, as measured by titration of infectious virus in HeLa cells. (c) *In situ* hybridization staining of lung sections from mice infected with RV-1B, RV-16 or UV-RV-1B. Top row, sections probed with antisense RNA probe to detect genomic viral RNA. Bottom row, sections probed with sense RNA probe to detect negative-strand replicative viral RNA. Sections probed with a respiratory syncytial virus M protein-specific probe³⁰ were negative (data not shown). Sections are representative of three mice per group. (d) Induction of antiviral IFNs α , β and λ in BAL, as measured by quantitative ELISA. (e) Frequency of IFN- γ -producing lung leukocytes in mice infected with RV-1B assessed at day 4 (left) or day 7 (right) after infection, cultured with or without RV-1B stimulation *ex vivo*. #### $P < 0.001$ comparing mice dosed with RV-1B to mice dosed with RV-16. At day 7, induction was virus-specific, +++ $P < 0.001$ for leukocytes stimulated *ex vivo* with RV-1B compared to unstimulated cells. (f) Rhinovirus-specific IgG measured by ELISA in serum collected at the indicated day after infection. All data are expressed as means \pm s.e.m., * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ for RV-1B compared with UV-inactivated control.

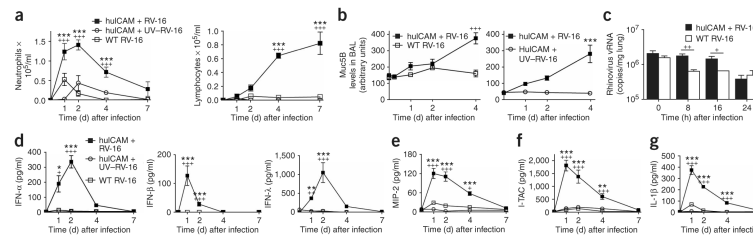


Figure 4.

Major group rhinovirus-16 infection of transgenic mice expressing a human/mouse ICAM-1 chimeric receptor. Groups of four human-mouse ICAM-1-expressing transgenic BALB/c mice were dosed intranasally with 5×10^6 TCID₅₀/ml RV-16 (huICAM + RV-16) or UV-RV-16 (huICAM + UV-RV-16); in addition, transgene-negative wild-type BALB/c mice were infected with RV-16 (WT RV-16). **(a)** Kinetic analysis of BAL cells showing neutrophils and lymphocytes at the indicated time points after infection, as assessed by differential cell counts. **(b)** Muc5B protein secretion in BAL, as measured by ELISA. Left, RV-16-infected transgenic mice compared with transgene-negative mice inoculated with RV-16. Right, transgenic mice inoculated with live or UV-RV-16. **(c)** Viral RNA (vRNA) in lung homogenate, as measured by qRT-PCR. **(d–g)** Production of IFN- α (**d**, left), IFN- β (**d**, center), IFN- λ (**d**, right), MIP-2 (**e**), I-TAC (**f**) and IL-1 β (**g**) in BAL, as measured by quantitative ELISA. All data are expressed as means (groups of seven for huICAM + RV-16 and groups of three or four for control groups) \pm s.e.m. * P < 0.05, ** P < 0.01 and *** P < 0.001 for huICAM + RV-16 groups compared to huICAM + UV-RV-16 controls. + P < 0.05, ++ P < 0.01 and +++ P < 0.001 for huICAM + RV-16 groups compared to transgene negative controls dosed with live rhinovirus-16.

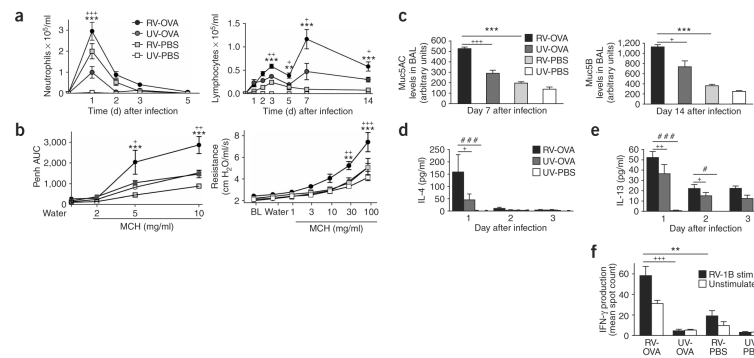


Figure 5.

Rhinovirus exacerbates airway inflammation, airway hyper-responsiveness, mucus production, and T_H1 and T_H2 cytokine responses in a model of acute allergic airway inflammation. OVA-sensitized mice were challenged intranasally with OVA or PBS control and infected with RV-1B (RV-OVA or RV-PBS) or UV-inactivated virus (UV-OVA or UV-PBS). (a) Kinetic analysis of BAL cells showing neutrophils (left) and lymphocytes (right) at the indicated time points after infection, as assessed by differential cell counts. (b) Airway hyper-responsiveness to increasing doses of methacholine (MCH), as assessed at 24 h after infection by Penh area under the curve (AUC) analysis (left). Penh results were confirmed by invasive measures of airway resistance (right). BL, baseline. (c) Muc5AC (left) and Muc5B (right) protein abundance in BAL, as determined by ELISA. Measurement of T_H2 cytokines IL-4 (d) and IL-13 (e) in BAL was performed by quantitative ELISA, and IFN- γ production by lung leukocytes (f) was assessed by ELISpot. All data are expressed as means \pm s.e.m. for groups of four mice. * P < 0.05, ** P < 0.01 and *** P < 0.001 RV-OVA compared to RV-PBS. + P < 0.05, ++ P < 0.01 and +++ P < 0.001 for RV-OVA compared to UV-OVA. # P < 0.05 and ### P < 0.001 for allergic groups (RV-OVA and UV-OVA) compared to controls (UV-PBS).